# Cationic and hydrolysable branched polymers by RAFT for complexation and controlled release of dsRNA

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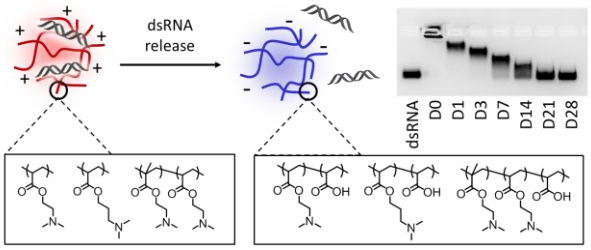
**TOC figure:**



## Abstract

The controlled release of nucleic acids from cationic polymers is an important criteria for the design of gene delivery systems, and can be difficult to achieve due to the persistent positive charges required to initially complex the nucleic acids. Here, we report the use of highly branched tertiary amine-rich polymers for the complexation and release of dsRNA over a prolonged period of time. Controlled release of dsRNA is obtained via hydrolysis of the polymer side chains and associated change in electrostatic charge. Reversible addition-fragmentation chain transfer (RAFT) polymerization was utilised to synthesise a series of branched polymers of 2-(dimethylamino)ethyl acrylate (DMAEA), 3-(dimethylamino)propyl acrylate (DMAPA), and 2-(dimethylamino)ethyl methacrylate (DMAEMA) (MW ~ 60,000 – 200,000 g/mol, and $Đ$ ~ 2 – 8) and copolymers thereof. The hydrolysis kinetics of all synthesised polymer materials were followed by $^{1}$H NMR spectroscopy. Complexation with dsRNA resulted in the formation of polyplex nanoparticles (N/P ratio of 5) with sizes of approximately 400 nm and surface charges of +15 mV. An agarose gel release study showed sustained release of dsRNA from p(DMAEA-*co*-DMAEMA) for a period of more than 2 weeks. Unlike branched PEI commonly used for gene delivery, the majority of these systems showed little toxicity to cells (NIH3T3 fibroblasts). The results point towards pDMAPA and p(DMAEA-*co*-DMAEMA) being promising polymers for the controlled release of oligonucleotides over prolonged periods.

## Introduction

Synthetic vectors that can be utilised to activate the RNA interference pathway, or transcribe for new proteins, by delivering nucleic acids (dsRNA, siRNA, mRNA, DNA) in target cells are gaining significant attention due to a number of potential advantages over viral vectors.[1] Particular benefits of synthetic vectors over viral vectors include: low immunogenicity,[2] high nucleotide loading,[3] as well as ease and reproducibility of production.[4] Yet, there are many barriers associated with gene-based therapies, which at the moment limit greatly the number of products successfully passing through clinical trials. These include degradation of nucleic acids by nucleases in physiological fluids as well as the short half-life of naked plasmid DNA *in vivo*, which was estimated to be in the region of 10 minutes post systemic injection.[1, 5] Synthetic vectors partly mediate these limitations by protecting nucleotides from enzymatic degradation, whilst also avoiding renal clearance and non-specific interactions with serum proteins and

extracellular compounds. In addition, synthetic vehicles need to extravasate from the vasculature, promote cell internalisation of otherwise negatively charged non-endocytosable nucleic acid biomolecules, and finally release the therapeutic nucleic acid in the intracellular environment. The latter is increasingly identified as a difficult hurdle to overcome as the strong electrostatic interactions between cationic synthetic systems and negatively charged oligonucleotides make it difficult to release the therapeutic cargo, dramatically decreasing the transfection yields.[6-8]

Thanks to advances in polymer and materials chemistry, stimuli responsive gene delivery systems have made significant progress in the last few years.[9, 10] These systems are able to respond to various stimuli and either trigger release of the transported nucleic acid, or promote endosomal escape of the carrier to the cell cytoplasm.[11] Examples of endogenous stimuli include: intracellular changes in pH environment,[12] change in redox environment,[13] temperature difference,[14] or the presence of enzymatic triggers;[15] while exogenous stimuli include: light,[16] ultrasound,[17] or even magnetism.[18]

A major shortcoming of these stimuli-responsive systems lies in the toxicity of the cationic polymers remaining after the oligonucleotide release. In response, increasing attention is being directed towards developing degradable or charge altering polymers with biocompatible by-products. Degradability can either be introduced in the polymer backbone, or through degradable polymer side chains. In the early 2000's, the Langer group prepared poly(β-amino esters) *via* Michael addition step growth polymerisation of diamines and diacrylates, resulting in cationic polymers with a degradable backbones and cationic groups for DNA complexation.[19, 20] In another example, Saltzman *et al*. encapsulated genetic material in poly(lactic-co-glycolic acid) (PLGA) to deliver siRNA efficiently through cervical mucus barrier in mice models.[21] Backbone degradable polymers based on oligo(carbonate-*b*-$\alpha$-amino ester)s were also shown to be efficient gene delivery vectors.[22] Introduction of polymer degradability through the polymer side chains, leading to biocompatible and non-toxic degradation products, has also been studied extensively by the group of Hennink among others.[23-25] The degradable side chain route however also has the added advantage of being able to incorporate a change in functionality and/or charge with side chain degradation. For example, polymers synthesised from 2-(dimethylamino)ethyl acrylate (DMAEA) have been investigated for the complexation and release of nucleic acids *via* hydrolysis of the ester connection between acrylate backbone and side chains.[26-28] Poly(DMAEA) proved to be an attractive polymer for non-viral gene delivery whose initial structure combines hydrolysable

side chains with cationic groups. Upon hydrolysis, cationic side chains turn into negatively charged acrylic acid moieties which enhance nucleic acid release *via* electrostatic repulsion. The self-catalysed hydrolysis of pDMAEA in water was initially reported to reach a limiting degree of hydrolysis of 60 to 70 % after one week in aqueous conditions at room temperature.[29] More recent studies confirmed that the hydrolysis and nucleic acid release occurs rapidly (1 to 10 hours[28]), and is consistent with a self-catalysed mechanism at a rate that is independent of pH, salt concentration, or any other external stimulus.[27, 30-32] A number of strategies have been employed to lengthen this release time, but it is yet to be extended past 72 hours.[33, 34]

Sustained release of nucleic acids for inducing RNA interference is acknowledged as a major challenge for the gene therapy field.[35-37] Here, we report prolonged protection and sustained release of dsRNA from synthetic polymers prepared via reversible addition-fragmentation chain transfer (RAFT) polymerisation. Highly branched polymers of 2-(dimethylamino)ethyl acrylate (DMAEA), 3-(dimethylamino)propyl acrylate (DMAPA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), and also statistical copolymers of DMAEA and DMAEMA were synthesised using di(ethylene glycol) diacrylate (DEGDA) or ethyleneglycol dimethacrylate (EGDMA) as cross-linkers. We attempted two different synthetic routes in order to slow the side chain hydrolysis of tertiary amine containing polymers. Firstly, by polymerising DMAPA, a monomer with a propyl chain rather than the ethyl of DMAEA. Secondly, by copolymerising DMAEA with DMAEMA, a non-hydrolysing equivalent monomer.[38] The branched polymers were characterized using multi-detector size exclusion chromatography (SEC) and their hydrolysis kinetics were followed with $^{1}$H NMR spectroscopy. Polyplex formation and dsRNA release was then investigated using agarose gel electrophoresis. Finally, polymer cytotoxicity was determined before and after side chain hydrolysis in relation to a model fibroblast cell line.

## Experimental

### Materials

2-(dimethylamino)ethyl acrylate (DMAEA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), 3-(dimethylamino)propyl acrylate (DMAPA), ethyleneglycol dimethacrylate (EGDMA), di(ethylene glycol) diacrylate (DEGDA), 4,4′-Azobis(4-cyanovaleric acid) (ACVA), 1,1′-Azobis(cyclohexanecarbonitrile) (VA088), polyethylenimine branched (bPEI, Mw ~25,000 by LS, Mn ~10,000 by SEC), Agarose, Ethidium bromide solution (500 μg/mL in $H_2O$), were all obtained from Sigma-Aldrich. All other materials were purchased from Fisher

Scientific, or Sigma-Aldrich. dsRNA was provided by Syngenta. 2-(((butylthio)-carbonothioyl)thio)propanoic acid (PABTC) was prepared according to a previously reported procedure.[39] (4-cyano pentanoic acid)yl ethyl trithiocarbonate (CPAETC) was prepared according to a previously reported procedure.[40] 50X Tris-Acetate-EDTA (TAE) buffer for gel electrophoresis was made up at concentration of 2.0M Tris acetate (Sigma Aldrich) and 0.05M EDTA (Sigma Aldrich) in deionised water, pH 8.2 - 8.4, stored at room temperature. Gel loading buffer for samples (colourless) was made up at 30% (vol/vol) glycerol (Sigma Aldrich) in deionised water and stored at room temperature. 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium salt), and Phenazine methosulfate (PMS) were obtained from Sigma.

**Characterisation**

Size Exclusion Chromatography (SEC) was performed in $CHCl_3$, using an Agilent 390-LC MDS instrument equipped with differential refractive index (DRI), viscometry, dual angle light scattering, and dual wavelength UV detectors. The system was equipped with 2 x PLgel Mixed D columns (300 x 7.5 mm) and a PLgel 5 μm guard column. The eluent was $CHCl_3$ with 2% TEA (trimethylamine) additive, and samples were run at 1 mL/min at 30 °C. Analyte samples were filtered through a nylon membrane with 0.22 μm pore size before injection. Apparent molar mass values ($M_{n,SEC}$ and $M_{w,SEC}$) and dispersity ($Đ$) of synthesized polymers were determined by DRI detector and conventional polystyrene (Agilent EasyVials) calibration and using Agilent SEC software. The absolute/true molecular weight ($M_{w,MALLS}$) and the intrinsic viscosity (IV) were determined by triple-detection SEC method using Agilent software and considering 100% polymer mass recovery (knowing the concentration). The Kuhn-Mark-Houwink-Sakurada parameter $\alpha$, relating to polymer conformation in solution was determined from the gradient of the double logarithmic plot of intrinsic viscosity as a function of molecular weight, using Agilent SEC software. Proton nuclear magnetic resonance spectra ($^1$H NMR) were recorded on a Bruker Advance 400 or 300 spectrometer (400 MHz or 300 MHz) at 27 °C, with chemical shift values (δ) reported in ppm, and the residual proton signal of the solvent used as internal standard. Proton-decoupled carbon nuclear magnetic resonance spectra ($^{13}$C NMR) were recorded on a Bruker Advance 400 (100 MHz) at 27 °C in $CDCl_3$, with chemical shift values (δ) reported in ppm, and the residual proton signal of the solvent used as internal standard (δC 77.16). Fourier transform infrared spectra (FTIR) were recorded on a Bruker Alpha FTIR ATR.

**Synthesis of branched acrylates pDMAEA and pDMAPA**

Conditions for polymerisations can be found in Supporting Information. For a typical polymerisation in which [M]: [DEGDA]: [PABTC]: [I] = 50: 2.5: 1: 0.1, PABTC (33.3 mg, 0.140 mmol), DMAEA (1 g, 6.98 mmol), DEGDA (74.8 mg, 0.35 mmol), ACVA (3.9 mg, 0.0140 mmol), and dioxane (1.19 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After a predetermined time, the solution was removed from the oil bath and the polymer precipitated in hexane (x3), and dried under vacuum. $M_{w,MALLS}$ = 299,000 g/mol, $Đ_{,RI}$ = 14 ($CHCl_3$ SEC, multi-detector). $^1$H NMR spectrum (400 MHz, $CDCl_3$, δ ppm): 4.15 (m, 2H, -C(O)O-C**H$_2$**-$CH_2$-$NMe_2$), 2.55 (m, 2H, -C(O)O-$CH_2$-C**H$_2$**-$NMe_2$), 2.27 (m, 6H, -$CH_2$-N**Me$_2$**), 2.11−0.91 (m, 3H, backbone). $^{13}$C NMR spectrum (100 MHz, $CDCl_3$, δ ppm): 174.28 (-**C**(O)O-), 62.33 (-C(O)O-**C**$H_2$-$CH_2$-), 57.52 (-**C**$H_2$-$N(CH_3)_2$), 45.71 (-N(**C**$H_3$)$_2$), 41.28 (backbone tertiary), 25.02 (backbone -**C**H2-). FTIR $\nu$ cm$^{-1}$: 2948 (medium, C-H alkane), 2821 and 2768 (medium, N-$CH_3$ amine), 1728 (strong, C=O ester), 1455 (medium, C-H alkane), 1251 (medium, C-N, amine), 1153 (strong, C-O ester).

**Synthesis of branched methacrylate pDMAEMA**

For a typical polymerisation in which [M]: [EGDMA]: [CPAETC]: [I] = 50: 0.95: 1: 0.025, CPAETC (33.5 mg, 0.127 mmol), DMAEMA (1 g, 6.36 mmol), EGDMA (24.0 mg, 0.121 mmol), VA-088 (0.777 mg, 0.00318 mmol), and dioxane (0.974 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 90 °C. After a predetermined time, the solution was removed from the oil bath and the polymer precipitated in hexane (x3), and dried under vacuum. $M_{w,MALLS}$ = 275,000 g/mol, $Đ_{,RI}$ = 8.2 ($CHCl_3$ SEC, multi-detector). $^1$H NMR spectrum (400 MHz, $CDCl_3$, δ ppm): 4.07 (m, 2H, -C(O)O-C**H$_2$**-$CH_2$-$NMe_2$), 2.57 (m, 2H, -C(O)O-$CH_2$-C**H$_2$**-$NMe_2$), 2.29 (m, 6H, -$CH_2$-N**Me$_2$**), 2.17−0.74 (m, 5H, backbone). $^{13}$C NMR spectrum (100 MHz, $CDCl_3$, δ ppm): 177.36 (-**C**(O)O-), 63.03 (-C(O)O-**C**$H_2$-$CH_2$-), 57.09 (-**C**$H_2$-$N(CH_3)_2$), 45.79 (-N(**C**$H_3$)$_2$), 44.73 (backbone quaternary), 19.01 (backbone -**C**$H_2$-), 18.78 (backbone -**C**$H_3$). FTIR $\nu$ cm$^{-1}$: 2944 (medium, C-H alkane), 2820 and 2769 (medium, N-$CH_3$ amine), 1722 (strong, C=O ester), 1455 (medium, C-H alkane), 1270-1265 (medium, C-N, amine), 1153 (strong, C-O ester).

**Synthesis of branched copolymer p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$)**

For a typical polymerisation in which [DMAEMA]: [DMAEA]: [EGDMA]: [CTA]: [I] = 40: 10: 1.5: 1: 0.05, CPAETC (21.06 mg, 0.0801 mmol), DMAEMA (0.503 g, 3.204 mmol),

DMAEA (0.115 g, 0.801 mmol), EGDMA (23.76 mg, 0.120 mmol), ACVA (1.12 mg, 0.0040 mmol), and dioxane (0.673 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After a predetermined time, the solution was removed from the oil bath and the polymer precipitated in hexane (x3), and dried under vacuum. $M_{w,MALLS}$ = 134,000 g/mol, $Đ_{,RI}$ = 3.1 ($CHCl_3$ SEC, multi-detector). $^1$H NMR spectrum (400 MHz, $CDCl_3$, δ ppm): 4.13 (m, 2H, -C(O)O-C**H$_2$**-$CH_2$-$NMe_2$), 2.69 (m, 2H, -C(O)O-$CH_2$-C**H$_2$**-$NMe_2$), 2.29 (m, 6H, -$CH_2$-N**Me$_2$**), 2.18−0.77 (m, 5H, backbone). $^{13}$C NMR spectrum (100 MHz, $CDCl_3$, δ ppm): 177.31 (-**C**(O)O-), 63.02 (-C(O)O-**C**$H_2$-$CH_2$-), 57.07 (-**C**$H_2$-$N(CH_3)_2$), 45.78 (-N(**C**$H_3$)$_2$), 44.70 (backbone tertiary), 20.02 (backbone -**C**$H_2$-), 18.02 (backbone -**C**$H_3$). FTIR $\nu$ cm$^{-1}$: 2944 (medium, C-H alkane), 2820 and 2769 (medium, N-$CH_3$ amine), 1722 (strong, C=O ester), 1455 (medium, C-H alkane), 1263-1270 (medium, C-N, amine), 1144 (strong, C-O ester).

**Synthesis of branched copolymer p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$)**

For a typical polymerisation in which [DMAEMA]: [DMAEA]: [DEGDA]: [CTA]: [I] = 10: 40: 1.5: 1: 0.05, CPAETC (21.06 mg, 0.0801 mmol), DMAEMA (0.126 g, 0.801 mmol), DMAEA (0.458 g, 3.204 mmol), DEGDA (25.68 mg, 0.120 mmol), ACVA (1.12 mg, 0.0040 mmol), and dioxane (0.713 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After a predetermined time, the solution was removed from the oil bath and the polymer precipitated in diethyl ether (x3), and dried under vacuum. $M_{w,MALLS}$ = 66,600 g/mol, $Đ_{,RI}$ = 1.7 ($CHCl_3$ SEC, multi-detector). $^1$H NMR spectrum (400 MHz, $CDCl_3$, δ ppm): 4.17 (m, 2H, -C(O)O-C**H$_2$**-$CH_2$-$NMe_2$), 2.65 (m, 2H, -C(O)O-$CH_2$-C**H$_2$**-$NMe_2$), 2.28 (m, 6H, -$CH_2$-N**Me$_2$**), 2.05−0.85 (m, 3H, backbone). $^{13}$C NMR spectrum (100 MHz, $CDCl_3$, δ ppm): $^{13}$C NMR spectrum (100 MHz, $CDCl_3$, δ ppm): 174.26 (-**C**(O)O-), 62.35 (-C(O)O-**C**$H_2$-$CH_2$-), 57.53 (-**C**$H_2$-$N(CH_3)_2$), 45.72 (-N(**C**$H_3$)$_2$), 41.07 (backbone tertiary), 25.02 (backbone -**C**$H_2$-). FTIR $\nu$ cm$^{-1}$: 2944 (medium, C-H alkane), 2820 and 2767 (medium, N-$CH_3$ amine), 1726 (strong, C=O ester), 1455 (medium, C-H alkane), 1263 (medium, C-N, amine), 1155 (strong, C-O ester).

**DLS/Zetapotential**

Dynamic light scattering measurements of resulting polymers and polyplexes at various N/P ratios were carried out using a Malvern NanoZS Zetasizer instrument (scattering angle of 173°,

10 mW He-Ne laser). For polyplex formation: appropriate amount of polymer stock solution and DNA stock solution were mixed and made up to a total volume of 1 mL in DI water (final concentration of polymer was 1 mg/mL, in all solutions). The resulting solutions were vortexed incubated for 30 minutes at room temperature and were analysed at 25°C. Each sample was run in triplicate and data was acquired using the software provided (Malvern Zetasizer). Zeta potential measurements were carried out of the same DLS samples at various N/P ratios using the same instrument, and Malvern disposable folded capillary cell (DTS1070) cuvettes.

**Agarose gel electrophoresis**

Agarose gels (1% w/v) were prepared with agarose and 1 × TAE buffer with DNAse/RNAse free water. The solution was cooled on the bench for 5 minutes and 100 µL of 0.5 µg/mL ethidium bromide solution was added. The mixture was poured into the casted agarose tray and a comb inserted. The gel was left to set for a minimum of 30 minutes at room temperature. The agarose gels were run in 1× TAE buffer. The final gel was visualized under UV illumination at 365 nm using a UVP benchtop UV transilluminator system. Polyplexes of dsRNA were prepared at various N/P ratios. dsRNA stock solution of 60 µg/mL was prepared sterile water, and polymer stock solution of 300 µg/mL. For polyplex formation: appropriate amount of polymer stock solution and dsRNA stock solution were mixed and made up to a total volume of 100 µL (final concentration of dsRNA was 0.030 µg/µL, in all solutions). Polyplexes were vortexed and incubated at room temperature for 30 minutes. Prior to loading, 30 µL of loading buffer was added to each sample and 20 µL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 20 minutes.

**Agarose gel dsRNA release study**

Polyplexes were formed in sterile water at an N/P ratio of 5 with a final concentration of 1 mg/mL dsRNA. Samples were then divided into separate microtubes for each sample time point and stored at room temperature, until the microtubes were frozen at the appropriate time. When all the time points has been collected, samples were defrosted diluted to 100 µg/mL dsRNA. Prior to loading, loading buffer was added to each sample and 20 µL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 20 minutes on a 1% agarose gel containing ethidium bromide.

**Cell culture**

NIH-3T3 mouse fibroblast cells were obtained from the Sigma-Aldrich and used between passages 5 and 15. Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of bovine calf serum, 1% of 2 mM glutamine and 1% penicillin/streptomycin. The cells were grown as adherent monolayers at 310 K under a 5% $CO_2$ humidified atmosphere and passaged at approximately 70–80% confluence.

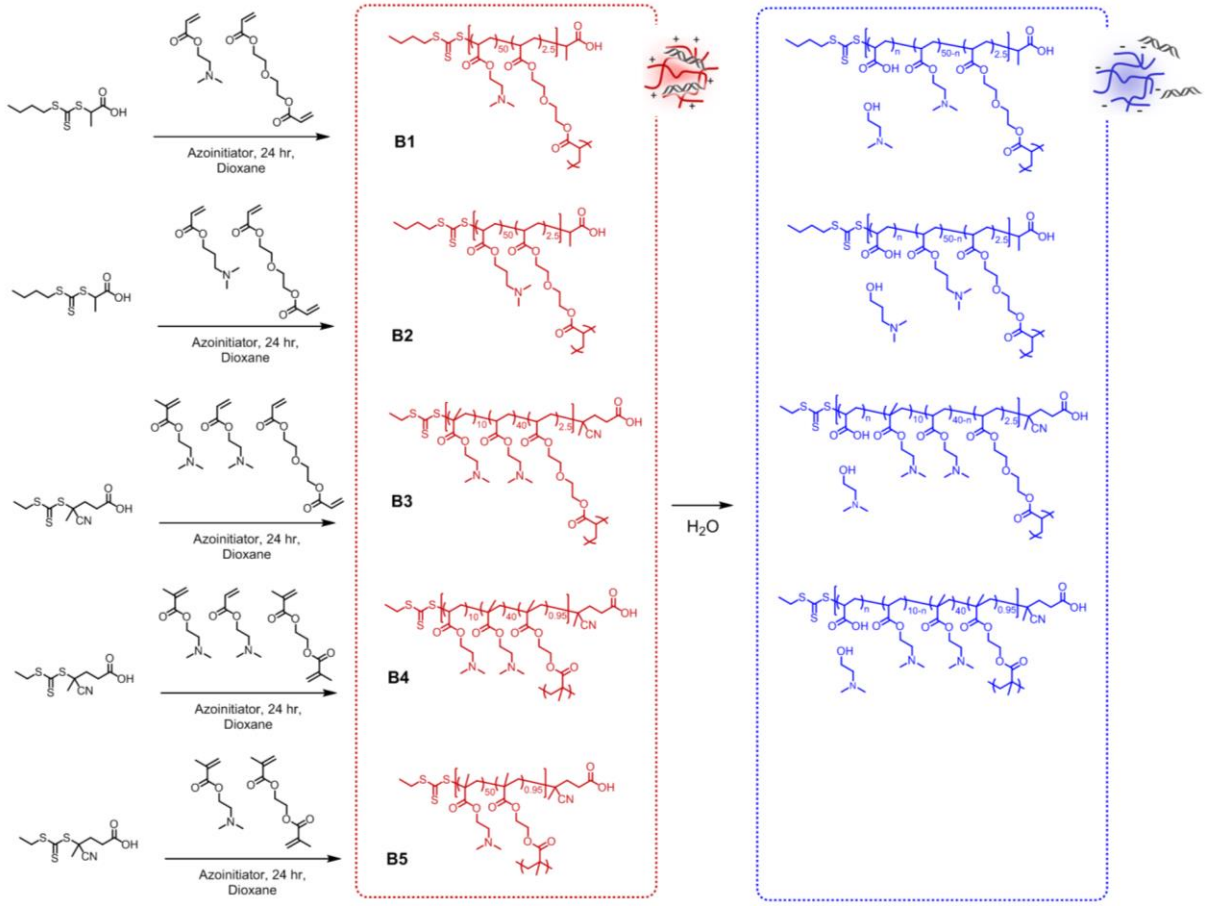
**In vitro toxicity assays**

NIH-3T3 mouse fibroblast cells were seeded in a 96 well plate at a density of $1 \times 10^4$ cells per well. After 16 hours, the culture medium was replaced by fresh media containing a series of dilution of the polymers. Following 24 hours incubation, the medium was removed and replaced with fresh medium. The cells were incubated with a freshly prepared solution of XTT (0.2 mg/mL) and N-methyl dibenzopyrazine methyl sulfate (250 µM) in medium for 16 hours. Absorbance of the samples was finally measured using a plate reader at 450 nm and 650 nm. The data presented are representative of a minimum of two independent experiments where each sample was measured in triplicate. Errors reported correspond to the standard deviation of the mean. For toxicity assays of polymers after two weeks hydrolysis, the polymers were incubated in sterile water (pH ~7.4) at concentrations of 8 mg/mL for two weeks. Before incubating with cells, hydrolysed polymer solution was diluted to the appropriate concentration in media.

## Results and Discussion

### Synthesis of highly branched polymers by RAFT

RAFT polymerisation was used to synthesise a series of highly branched polymers from a range of tertiary amine-containing vinyl monomers and divinyl branching comonomers (**Scheme 1**). Reaction temperature, initiator, chain transfer agent (CTA), and branching comonomer, were varied depending on monomer composition. Acrylate monomers (DMAEA, DMAPA and DEGDA) were polymerised at 70 ºC using PABTC as CTA, and ACVA as initiator. Methacrylate monomers (DMAEMA and EGDMA) were polymerised at 90 ºC using CPAETC as CTA, and VA088 as initiator. Copolymers of acrylate and methacrylate were polymerised at 70 ºC using CPAETC as CTA and ACVA as initiator. Either EGDMA or DEGDA was used as branching comonomer depending on the major proportion of monomer being acrylate or methacrylate. Having a branching monomer with similar reactivity as the main monomer feed increases its insertion into the backbone during the propagation step. Based on previous work, a degree of polymerisation (DP) of 50 with 0.95 – 2.5 molecules of branching monomer per CTA was targeted in order to form soluble highly branched polymers with similar and high molecular weights but without gelation.[41] Linear equivalents of the branched DMAEMA/DMAEA copolymers were also synthesised, in order to identify any possible differences of polymer architecture on rate of hydrolysis.



***Scheme 1.*** *Synthesis of highly branched polymers (**B1-B5**)* via *RAFT polymerisation of tertiary amine-containing monomers (DMAEA, DMAPA, and DMAEMA) and divinyl branching monomers along with decomposition products from hydrolysis in aqueous/physiological conditions.*

SEC traces for the resulting branched polymers show a broad molecular weight distribution, as expected for branched polymerisation systems (**Figure 1a**). The molecular weights of the polymers were determined to be between 100,000 – 200,000 g/mol using light scattering detection SEC, as seen in **Table 1**. Information about the branched nature of the polymers was extracted from the intrinsic viscosity values measured by the viscometry detector. The Kuhn-Mark-Houwink-Sakurada $\alpha$ values for each polymer were calculated from plotting the logarithmic intrinsic viscosity against the logarithmic molecular weight ($\alpha$ = gradient) (**Figure 1b**). An $\alpha$ value between 0.6 and 0.8 corresponds to random coil in a good solvent whereas lower values indicates more dense structures close to the hard sphere model typically used for branched or star architectures. Table 1 shows the $\alpha$ values obtained for the synthesised polymers. For linear polymers $\alpha$ values fall between 0.6 – 0.8. The synthesised branched polymers had $\alpha$ values of between 0.36 – 0.53 as expected for more dense globular structures. $^1$H NMR spectra of the purified polymers are shown in **Figure 1c**, however because of the branched nature of the polymers, and non-visible end groups, it was not possible to determine molecular weight values from the NMR spectra.

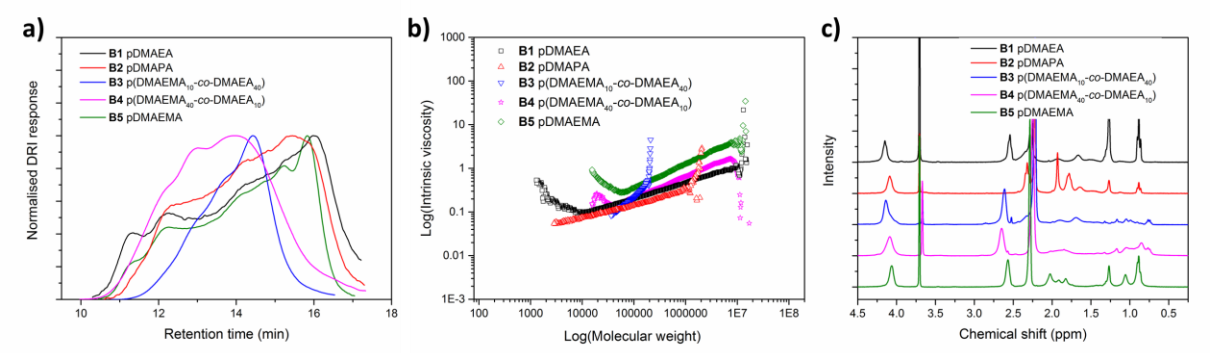



***Figure 1. a)*** *Size-exclusion chromatograms (normalised DRI detector response vs retention time) for the branched polymers;* ***b)*** *Kuhn-Mark-Houwink-Sakurada (KMHS) plots of log intrinsic viscosity against log molecular weight from SEC viscosity detector in $CHCl_3$ eluent;* ***c)*** *$^1$H NMR spectra of tertiary amine containing highly branched polymers in deuterated chloroform.*

***Table 1.*** *Characterisation of branched and linear polymers prepared in this study, including compositions, molecular weights, dispersity, and Kuhn-Mark-Houwink-Sakurada alpha values.*

| Sample | Structure | M1 : M2 : M3 : B : CTA [a] | $M_{n,SEC}$ (g/mol) [b] | $M_{w,SEC}$ (g/mol) [b] | $Đ$ [b] | $M_{w,MALLS}$ (g/mol) [c] | $\alpha$ [d] |
|---|---|---|---|---|---|---|---|
| **B1** | p(DMAEA$_{50}$-*co*-DEGDA$_{2.5}$) | 0 : 50 : 0 : 2.5 : 1 | 19,000 | 268,000 | 14 | 299,000 | 0.36 |
| **B2** | p(DMAPA$_{50}$-*co*-DEGDA$_{2.5}$) | 0 : 0 : 50 : 2.5 : 1 | 22,000 | 168,000 | 7.6 | 193,000 | 0.53 |
| **B3** | p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$-*co*-DEGDA$_{1.5}$) | 10 : 40 : 0 : 1.5 : 1 | 18,100 | 31,100 | 1.7 | 66,600 | 0.53 |
| **B4** | p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$-*co*-EGDMA$_{1.5}$) | 40 : 10 : 0 : 1.5 : 1 | 17,600 | 54,900 | 3.1 | 134,000 | 0.42 |
| **B5** | p(DMAEMA$_{50}$-*co*-EGDMA$_{0.95}$) | 50 : 0 : 0 : 0.95 : 1 | 27,000 | 218,000 | 8.2 | 275,000 | 0.41 |
| **L1** | p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) | 10 : 40 : 0 : 0 : 1 | 7,300 | 9,000 | 1.24 | 8,200 | 0.56 |
| **L2** | p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$) | 40 : 10 : 0 : 0 : 1 | 7,100 | 9,800 | 1.37 | 8,400 | 0.61 |

[a] Ratio of monomer 1 (DMAEMA) to monomer 2 (DMAEA) to monomer 3 (DMAPA) to brancher (EGDMA or DEGDA) to CTA. [b] From $CHCl_3$ SEC, DRI detector, linear PS standard. [c] Molecular weight from light scattering detection on $CHCl_3$ SEC. [d] $\alpha$ = Kuhn-Mark-Houwink-Sakurada parameter, from $CHCl_3$ SEC viscometry detector.

## Determination of DMAEMA and DMAEA reactivity ratios

To better understand the copolymerisation of DMAEA and DMAEMA, the kinetics of their polymerisation was studied using $^1$H NMR spectroscopy. A statistical copolymer of DP 50 containing 50% DMAEA and 50% DMAEMA was targeted (see polymerisation conditions in Supplementary Information). The conversion rate of each monomer, reported in **Figure 2a**, shows that the methacrylate is incorporated into the polymer faster than the acrylate. This trend, expected in the case of acrylate and methacrylate copolymerisations, could lead to slight gradient like nature of the polymer.[42] For example, differences in monomer reactivity ratios in the copolymerisation of butyl acrylate and methyl methacrylate of rBA = 0.36 and rMMA = 2.07 (rBA x rMMA = 0.75) lead to a copolymer with a higher proportion of MMA incorporation in the beginning of the polymerisation.[43]

To further investigate the possible gradient-like nature of the copolymers of DMAEA and DMAEMA, the reactivity ratios of this comonomer system have been determined in typical RAFT conditions using three different calculation methods based on the same experimental data. For this purpose, a series of copoplymers containing 10%, 30%, 50%, 70%, and 90% of DMAEA were prepared. DP 50 was targeted and the polymerization were stopped at less than 10% total monomer conversion. The reactivity ratios were then calculated using the Fineman

Ross method (rDMAEMA = 2.55 and rDMAEA = 0.93), the Kelen Tudos linearisation method (rDMAEMA = 2.29, rDMAEA = 0.71), and using a non-linear regression method based on a data fit of the copolymer equation (rDMAEMA = 2.13, rDMAEA = 0.69) (see Supporting Information). The last method, generalised by Van Herk in the 1990's, is considered to be more accurate as it does not rely on linearisation of the data.[44, 45] **Figure 2b** shows the plot of monomer incorporation ratio against monomer feed ratio and the non-linear regression fit of the data which gives values for the reactivity ratios. All three methods give values which are in good agreement, and indicate that DMAEMA has a tendency for self-propagation. The multiplication of reactivity ratios (rDMAEMA x rDMAEA = 1.47) indicates a slight gradient tendency in the polymerisation.[46] However, we reason that when many chains (DP = 50) with slight monomer gradient are linked together in a statistical manner (as in the branched polymer synthesis), the effect of the gradient in the branched copolymers is greatly reduced.

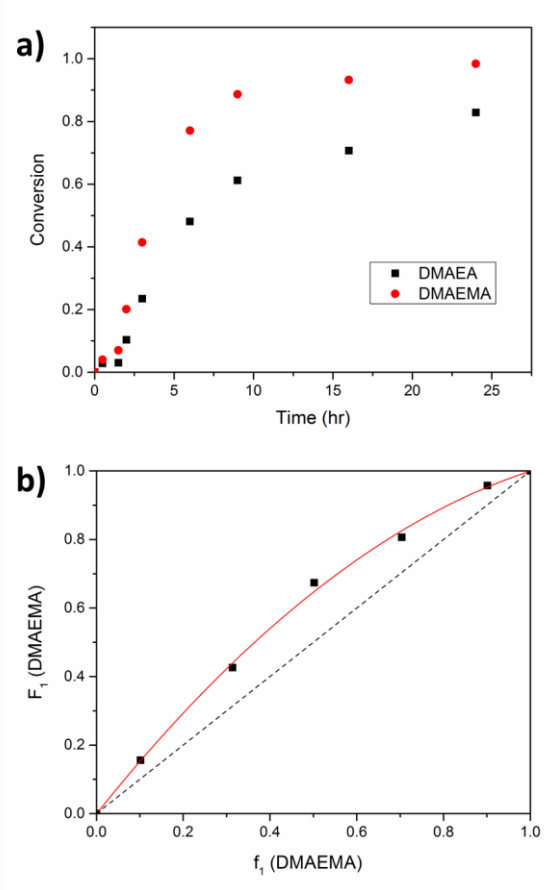



***Figure 2. a)*** *Monomer conversion against time for a statistical copolymer of DP = 50 containing 50 % DMAEA and 50 % DMAEMA, as determined by $^1$H NMR spectroscopy;* ***b)*** *Monomer incorporation ratio ($F_1$) against monomer feed ratio ($f_1$) (dots), including the non-linear regression fit of the data (red curve, giving rDMAEMA = 2.13, rDMAEA = 0.69), dashed line represents $F_1 = f_1$.*

**Polymer hydrolysis kinetic study**

The self-catalysed hydrolysis of pDMAEA in water was first reported in 1989, and was found to reach a limiting degree of hydrolysis of ~ 60 or 70 % after one week in aqueous conditions at room temperature.[29] More recent studies have also found the hydrolysis to occur rapidly, and to be consistent with a self-catalysed mechanism at a rate that is independent of pH, salt concentration, or any other external stimulus.[27, 30-32] The hydrolysis kinetics of the branched tertiary amine-containing polymers were studied using $^1$H NMR spectroscopy in $D_2O$ (pH ~ 7.4). **Figure 3a** shows the $^1$H NMR spectra of branched p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) over a period of 20 days. $^1$H NMR spectra of hydrolysis study of remaining polymers can be found in the Supporting Information. The hydrolysis of DMAEA units in the polymer results in the appearance of sharp peaks at ~3.7 ppm, 2.9 ppm, and 2.6 ppm, due to the creation of dimethylaminoethanol hydrolysis product. Integration of these peaks in comparison with the peak at 4.2 ppm representing the total sum of monomer units was used to calculate the percentage hydrolysis. The resulting hydrolysis kinetic profiles are presented in **Figure 3b**. Branched pDMAEA hydrolyses relatively fast at first (40 % hydrolysed after 17 hours), then slows down to reach approximately 70 % hydrolysis after 20 days. As expected,[38] the methacrylate polymer pDMAEMA does not shown any significant sign of hydrolysis after 20 days. While branched p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) hydrolyses with a similar initial rate as pDMAEA homopolymer, the presence of 20% of methacrylate units resulted in an hydrolysis rate that is relatively similar to pDMAEA homopolymer in the first few hours, but which only reaches 50 % hydrolysis after 20 days. Branched pDMAPA reached a similar value of around 50 % hydrolysis after 20 days, but with a slower initial gradient. Branched copolymer p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$) showed very little hydrolysis and reaches a plateau of around 5 % hydrolysis after 3 days.

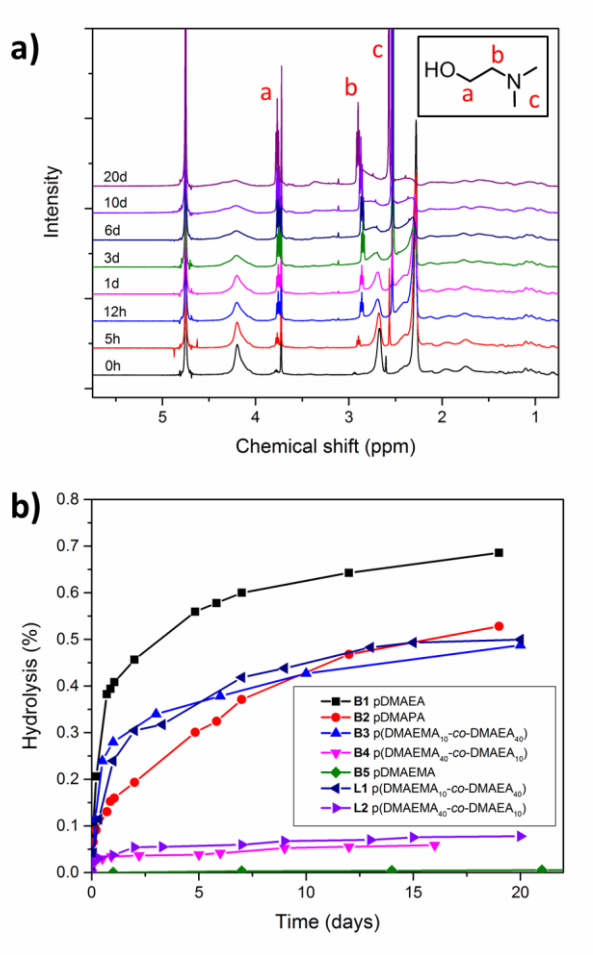



***Figure 3. a)*** *Hydrolysis of branched p(DMAEMA$_{10}$-co-DMAEA$_{40}$) in $D_2O$ (pH ~7.4) as determined using $^1$H NMR spectroscopy; (other polymers shown in Supp. Info);* ***b)*** *Hydrolysis kinetics of synthesised branched and linear polymers in $D_2O$ (pH ~7.4) determined using $^1$H NMR spectroscopy.*

We hypothesise that the hydrolysis of DMAEA in methacrylate /acrylate copolymers could be slowed by the more hydrophobic nature of the polymer backbone associated with the presence of methacrylate repeating units. This is exemplified by the copolymers containing 20% DMAEA (**B4** and **L2**) proceeding to 5% hydrolysis instead of the expected value of around 15-20%.

It is worth noting that hydrolysis of 50% of the side chains of these polymers would result in zwitterionic polymers with an overall neutral charge, thus losing their ability to complex negatively charge dsRNA. Therefore, the two systems reaching 50% hydrolysis after a period of weeks, both p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) and pDMAPA, were thought to be promising candidates for slow dsRNA release.

## Polyplex formation and dsRNA release

Complexation of the branched polymers with dsRNA to form polyplexes was characterised by DLS and zetapotential measurements, as well as agarose gel electrophoresis. The later technique showed complete complexation of dsRNA by all the branched polymers for N/P ratios of either 1 or 2 (**Figure S7**). Therefore, an N/P ratio of 5 was chosen to characterise polyplex physiochemical properties in terms of size and surface charge (**Table 2**).

***Table 2.*** *Size and surface charge (zetapotential) of polyplexes formed by complexation of dsRNA with polymers at N/P = 5, as measured by dynamic light scattering and electrophoretic light scattering.*

| Polyplex (N/P 5) | Zetapotential (mV) | Size by number (nm) | PDI |
|---|---|---|---|
| **B1** pDMAEA | +15.1 ±1.4 | 488.9 ±87.1 | 0.53 |
| **B2** pDMAPA | +14.8 ±1.3 | 456.8 ±123.1 | 0.41 |
| **B3** p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) | +15.3 ±1.5 | 448.4 ±65.3 | 0.85 |
| **B4** p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$) | +15.8 ±1.3 | 336.9 ±45.8 | 0.55 |
| **B5** pDMAEMA | +15.0 ±1.3 | 399.7 108.9 | 0.33 |
| **L1** p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) | +14.3 ±1.3 | 541.1 ±100.6 | 0.58 |
| **L2** p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$) | +15.7 ±1.4 | 488.7 ±90.5 | 0.47 |

All synthesised polymers appeared to form polyplexes with an overall positive charge of between 14 to 16 mV. Polyplex sizes were also found to be similar, with most polyplex diameters measured between 400 and 500 nm. The polydispersity values (**Table 2**) indicate a broad distribution of sizes for the polyplexes in solution. It is worth noting that this set of values represents a crude estimation of the real size of the polyplexes, as measurement by DLS is subject to significant error when assuming spherical shape.

Next, an agarose gel electrophoresis experiment was designed to correlate polymer side chain hydrolysis and resulting charge reversal of the polymers to an observable release of dsRNA. Polyplexes were formed in sterile water at an N/P ratio of 5, and divided into separate microtubes for each sample time point, following which the samples were frozen at the appropriate time before being simultaneously analysed by agarose gel electrophoresis.

Pictures of the gels are shown in **Figure 4**. When the dsRNA is bound in a positively charged polyplex nanoparticle, the dsRNA band can be seen at the top of the gels in the well. Whereas negatively charged unbound dsRNA will move through the gel, resulting in a band at the bottom of the gel. It can be seen that all the synthesised polymers form strongly bound dsRNA polyplexes on day 0, apart from branched pDMAEA homopolymer which has already started to release the dsRNA over the course of sample preparation and/or gel preparation (**Figure 4a**). Both linear and branched p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) (**Figures 4b** and **4c** respectively) start to release dsRNA after one day, and continue to release dsRNA over the course of more than 14 days as the polymer slowly hydrolyses and the band corresponding to dsRNA moves down the gel towards the location of uncomplexed dsRNA. For these samples, complete dissociation from the dsRNA is observed after 21 days. Branched p(DMAPA) homopolymer showed a slower initial release (**Figure 4d**) with dsRNA remaining complexed for three days prior to releasing dsRNA. Full dissociation of dsRNA from the polymer was observed after 14 days. Both linear and branched p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$) show no dsRNA release over the course of the study (**Figure 4e** and **4f**), which is consistent with the very low percentage of hydrolysis for these polymers. Accordingly, branched pDMAEMA homopolymer, which does not undergo hydrolysis of the side chains, showed no dsRNA release.

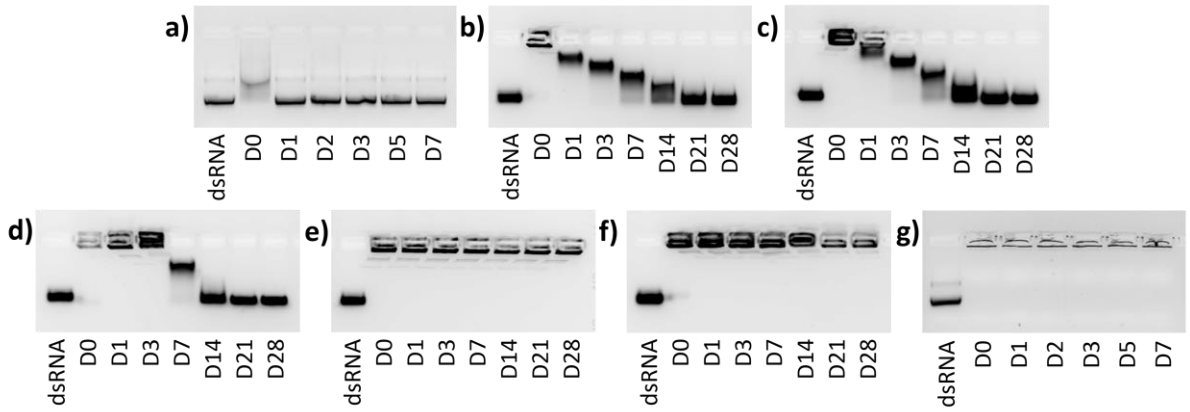



***Figure 4.*** *Agarose gel electrophoresis assay of branched cationic polymer – dsRNA polyplex nanoparticles (all at N/P ratio 5) over time periods up to 28 days;* ***a)*** *branched pDMAEA;* ***b)*** *linear p(DMAEMA$_{10}$-co-DMAEA$_{40}$);* ***c)*** *branched p(DMAEMA$_{10}$-co-DMAEA$_{40}$);* ***d)*** *branched pDMAPA;* ***e)*** *linear p(DMAEMA$_{40}$-co-DMAEA$_{10}$);* ***f)*** *branched p(DMAEMA$_{40}$-co-DMAEA$_{10}$);* ***g)*** *branched pDMAEMA.*

Overall, the results appear in accordance with the hydrolysis kinetic profiles of the polymers, and confirm the potential of the synthesised polymers for gene delivery applications. Branched

pDMAPA shows potential for delayed gene delivery applications, where release of oligonucleotides is required approximately one week after application. To the best of our knowledge, this study is the first to characterise the hydrolysis of pDMAPA and demonstrate its use for oligonucleotide complexation and controlled release.

Copolymers of DMAEA and DMAEMA appear to be better suited for extended release of oligonucleotides, as they were shown to release dsRNA from day 1 to past day 14. We attribute this release profile to the presence of non-hydrolysable DMAEMA groups, which remain positively charged throughout the degradation process. These polymers show dsRNA release for over two weeks, which is a significant improvement over the pDMAEA and pDMAPA release profiles. Such sustained dsRNA release profiles would be useful for single injection gene silencing over periods of weeks.

**Polymer cytotoxicity**

The hydrolysis of cationic pDMAEA into biocompatible and non-cytotoxic poly(acrylic acid) (pAA) and N,N-dimethylamino ethanol (DMAE) is of benefit for use in biomedical appllications. PAA has been shown to be non-toxic to mammalian cell lines both *in vitro* and also *in vivo*,[47, 48] while DMAE is approved and safely used in the cosmetics and the neutraceutical industries. Toxicity of synthesised polymers was investigated before and after hydrolysis against a model cell line, NIH-3T3 fibroblast. Branched polyethylenimine (bPEI), commonly used for gene delivery purposes, was also included as a positive control. **Figure 5a** shows the relative percentage of viable cells following 24 hours incubation with the polymers before hydrolysis. Branched pDMAEMA and p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$) showed a toxicity profile similar to that of bPEI, with complete death of the cells at concentrations above 50 μg/mL. Branched pDMAPA and p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) showed a less toxic profile with zero cell survival at concentrations above 200 μg/mL and 2 mg/mL, respectively. In contrast, the other polymers appeared to be relatively non-cytotoxic under the conditions tested. The trend of polymer toxicities appear to match the trend of hydrolysis profiles with the more hydrolysable polymers being less toxic. This can be attributed to the conversion of potentially toxic cationic polymers into biocompatible poly(acrylic acid) over the time of the experiment, or the increased membrane activity of the more hydrophobic methacrylate containing polymers.

**Figure 5b** shows the toxicity profiles determined for the same polymers but after a 2 week pre-incubation in water at room temperature, following which polymers are expected to be almost completely hydrolysed. As expected, the non-hydrolysable polymers pDMAEMA and bPEI, have similar toxicity profiles to before incubation in water. The branched copolymer with majority DMAEMA monomer, p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$), showed a slightly reduced toxicity compared to the non-hydrolysed version, which can be attributed to the hydrolysis of the small amount of DMAEA units. All other polymers tested showed no toxicity under the conditions studied, illustrating the conversion from toxic cationic polymer to non-toxic pAA.

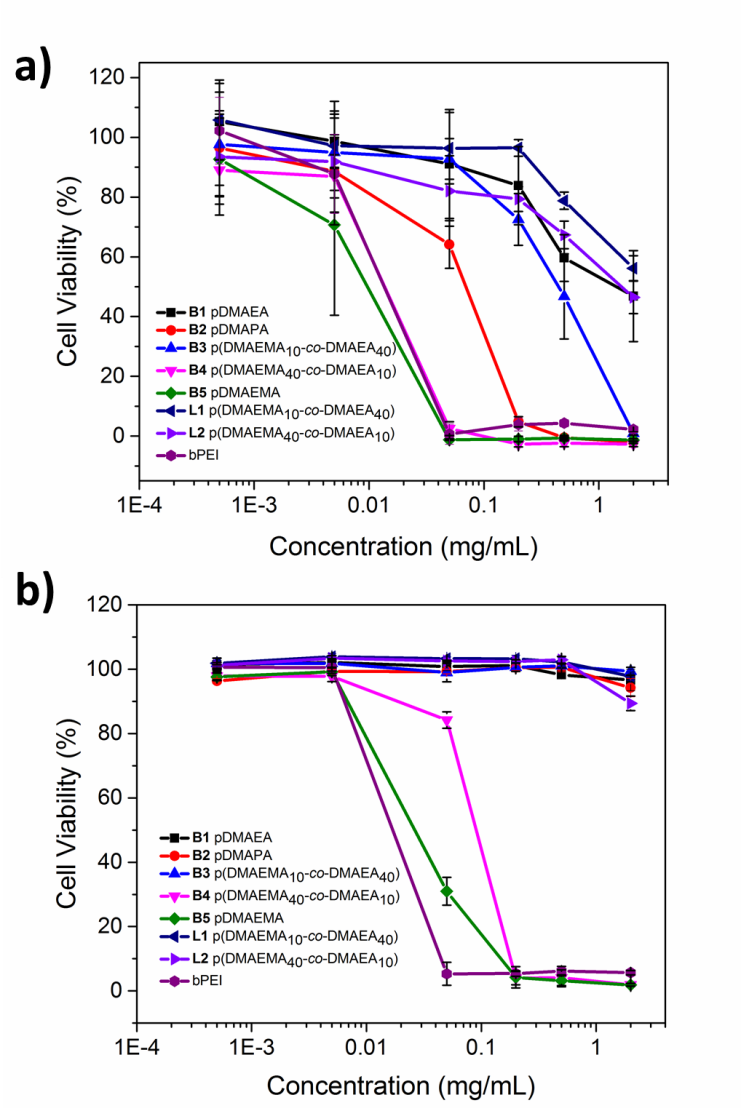



***Figure 5.*** *NIH-3T3 cell viability following 24 hr incubation in the presence of linear and branched polymers, as determined using XTT assay;* ***a)*** *initial polymers;* ***b)*** *polymers pre-incubated for 2 weeks in $D_2O$ at pH ~ 7.4.*

## Conclusions

Highly branched polymers were synthesised with RAFT polymerisation of hydrolysable acrylate monomers containing tertiary amine functionality (DMAEA, DMAPA), non-hydrolysable methacrylate counterpart (DMAEMA), and branching divinyl comonomers (EGDMA and DEGDA). The highly branched nature of these polymers was characterised using multi-detector SEC, and the copolymers were found to have a slightly gradient nature from calculation of the monomer reactivity ratios of the DMAEA and DMAEMA. Hydrolysis kinetics of the polymers were studied with $^{1}$H NMR spectroscopy, and showed pDMAPA and p(DMAEMA-*co*-DMAEA) to have hydrolysis profiles favourable for extended release of dsRNA compared to the fast hydrolysing pDMAEA. All the materials were shown to form polyplexes in presence of dsRNA and were characterized by DLS and agarose electrophoresis gels. The dsRNA release profiles of polyplex nanoparticles were also determined using agarose gel electrophoresis and both branched pDMAPA and p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$) showed excellent prolonged release of oligonucleotide in aqueous conditions. Finally, the polymer cytotoxicity to NIH-3T3 fibroblast cell line was determined both before and after side chain hydrolysis. The most promising materials, pDMAPA and p(DMAEMA$_{10}$-*co*-DMAEA$_{40}$), show negligible toxicity in the appropriate concentration range (50 - 200 μg/mL) even before complete hydrolysis to biocompatible p(acrylic acid). Whereas non-hydrolysable branched pDMAEMA, bPEI, and also branched p(DMAEMA$_{40}$-*co*-DMAEA$_{10}$), had significant toxicity with zero cell proliferation above concentrations of 50 μg/mL. These polymeric materials show great potential for therapeutic nucleic acid delivery as polyplexes, but could also represent an improved material for hydrogel formulations, nucleic acid releasing films, or other implantable or injestable polymeric constructs for controlled release.

## Acknowledgments

The Royal Society Wolfson Merit Award (WM130055; S.P.), Monash-Warwick Alliance (S.P.), and Syngenta (A.C., R.W.) are acknowledged for financial support. The European Research Council (TUSUPO 647106; S.P., R.P.) and the German Research Foundation (DFG, GZ: HA 7725/1-1; M.H.) are gratefully acknowledged for funding. The authors would like to thank Dr Daniel Lester and the Polymer Characterisation Research Technology Platform for use of GPC facilities.

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